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Review

Therapeutic glycoprotein production in mammalian cells

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ABSTRACT

Over the last years, the biopharmaceutical industry has significantly turned its biologics production towards mammalian cell expression systems. The presence of glycosylation machineries within these systems, and the fact that monoclonal antibodies represent today the vast majority of new therapeutic candidates, has largely influenced this new direction. Recombinant glycoproteins, including monoclonal antibodies, have shown different biological properties based on their glycan profiles. Thus, the industry has developed cell engineering strategies not only to improve cell's specific productivity, but also to adapt their glycosylation profiles for increased therapeutic activity. Additionally, the advance of "omics" technologies has recently given rise to new possibilities in improving these expression platforms and will significantly help developing new strategies, in particular for CHO (Chinese Hamster Ovary) cells.

1. Introduction

Over the past decade, more than a hundred new biopharmaceutical products have been approved and marketed in the United States (US) and European Union (EU). Market value for these biologics was recently estimated at $140 billion US, with a total of over two hundred therapeutics (Walsh, 2014). A significant portion of these products are recombinant proteins, with an ongoing increase in the number of them produced in mammalian expression platforms (Walsh, 2014). This trend is mostly driven by the increased attention directed to post-translational modifications of these biologics, in particular towards their glycosylation state. Indeed, several efforts have been made over the last few years to understand how glycosylation can influence the biological activity of therapeutics. Studies have demonstrated that proper glycosylation profiles can improve recombinant protein properties such as increase their stability and half-life in blood circulation and decrease their immunogenicity (Ashwell and Harford, 1982; Runkel et al., 1998; Ghaderi et al., 2010; Ghaderi et al., 2012; Jefferis, 2016a; Jefferis, 2016b; Kuriakose et al., 2016).

Among the mammalian-based expression systems, CHO cells is by far, the most commonly used cell line. It is involved in the production of over 70% of recombinant biopharmaceutical proteins, most of them being monoclonal antibodies (mAbs) (Durocher and Butler, 2009; Kim et al., 2012; Butler and Spearman, 2014). This review will summarize the recent advances in production of glycoproteins in mammalian cells, with a particular emphasis on the CHO cell system. The various expression systems currently used for therapeutic glycoprotein production (Fig. 1) will be overviewed and cell engineering strategies used to improve biologics production and/or quality will be discussed. Finally, we will also describe the different "omics" approaches used lately in the field in order to improve glycoprotein production and/or glycosylation.

Abbreviations: EU, European Union; FDA, Food and Drug Administration; EMA, European Medicines agency; HEK, human embryonic kidney; shRNA, short hairpin RNA; siRNA, small interference RNA; α-gal, galactose-α1,3-galactose; Neu5Gc, *N*-glycolylneuraminic acid; IgG, Immunoglobulin G; GS, glutamine synthase; KO, knock out; DHFR, dihydrofolate reductase; MSX, methionine sulfoximine; MTX, methotrexate; SILAC, stable isotope labeling with amino acids in cell culture; iTRAQ, isobaric tags for relative and absolute quantification; Fc, fragment crystallizable; mAbs, monoclonal antibodies; cDNA, coding DNA; BHK, Baby Hamster Kidney; RMCE, recombination-mediated cassette exchange; ZFN, zinc finger nuclease; TALEN, transcription activators like effectors nucleases; NHEJ, non-homologous end joining; HDR, homologous-directed recombination; HPRT, hypoxanthine phosphoribosyltransferase; CRISPR, clustered regularly interspaced short palindromic repeats; ACE, artificial chromosome expression; S/MAR, scaffold/matrix attachment region; UCOE, ubiquitously acting chromatin opening elements; STAR, stabilizing and anti-repressor; CMV, cytomegalovirus; mTOR, mechanistic target of rapamycin; NaB, sodium butyrate; ER, endoplasmic reticulum; EPO, erythropoietin; IFNγ, interferon gamma; t-PA, tissue plasminogen activator; SNARE, *N*-ethylmaleimide-sensitive factor attachment protein receptors; ADCC, antibody-dependent-cell-mediated cytotoxicity; GnTIII, beta-1,4-mannosyl-glycoprotein 4-beta-*N*-acetylglucosaminyltransferase; ManNAc, *N*-Acetyl-D-mannosamine; NGS, next generation sequencing; NeuNAc, N-acetylneuraminic acid; TCA, tricarboxylic acid; FcγR, Fc-gamma receptor; ManII, mannosidase II

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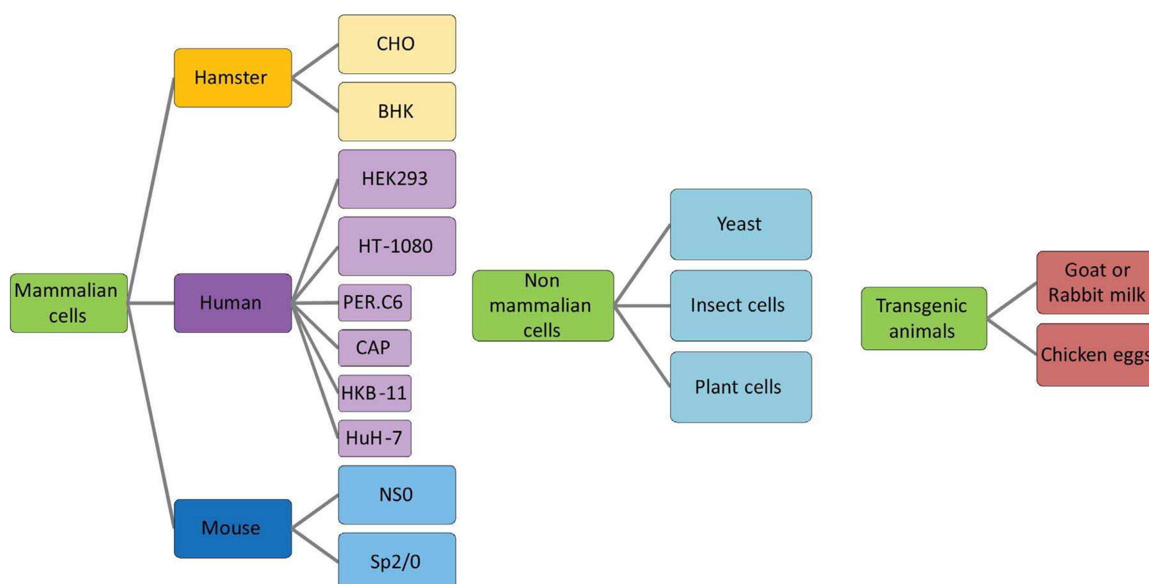

Fig. 1. Expression systems used for glycoprotein production by biopharmaceutical industries.

2. Cell hosts

2.1. Chinese hamster ovary cells

CHO cells are widely used for glycoprotein production because of their numerous advantages. These cells can achieve substantial production rate, are suitable for large-scale industrial suspension culture and can be adapted to grow in various serum-free and chemically defined culture media (Kim et al., 2012; Lai et al., 2013). Since CHO cells produce recombinant glycoproteins with human-like glycans, the generated products are to more likely be compatible and bioactive within human hosts (Kim et al., 2012; Lai et al., 2013). Furthermore, these cells are refractory to infection by human viruses, which minimizes biosafety risks for commercial production purpose (Boeger et al., 2005). This decreased susceptibility could be attributed to the fact that many viral entry genes are not expressed in CHO cells (Xu et al., 2011). Moreover, different gene amplification systems have been developed and used in CHO cells, which allow for high titer yields and good specific productivity (Durocher and Butler, 2009; Kim et al., 2012; Lai et al., 2013). There are many examples of biotherapeutic glycoproteins approved by the Food and Drug Agency (FDA) and the European Medicines Agency (EMA) currently produced in these cells. Several monoclonal antibodies such as Siltuximab (SYLVANT®), Pertuzumab (PERJETA®) and Rituximab (RITUXAN®), as well as other proteins such as tissue plasminogen activator (tPa, ACTILYSE®, ACTIVASE®) and Human DNAse (PULMOZYME®) are just some of the many examples of biotherapeutics generated in CHO cells (for a recent list see (Dumont et al., 2015)). In 2015, more than half of the thirteen new biologics approved were recombinant proteins produced in CHO cells (Sellick et al., 2011a). Among these products, four monoclonal antibodies, Daratumumab (DARZALEX®), Mepolizumab (NUCALA®) and Evolocumab/Alirocumab (REPATHA®/PRALUENT®) are used to treat multiple myeloma, asthma and hypercholesterolemia, respectively. The same trend is currently observed in 2016, where again more than half of the approved biotherapeutics are produced in CHO cells (FDA, 2016). Although CHO cells possess many advantages for glycoprotein productions, they are unable to produce some types of human glycosylation, such as α-2,6-sialylation and α-1,3/4-fucosylation (Patnaik and Stanley, 2006). Moreover, CHO cells produce glycans that do not occur in human cells, namely *N*-glycolylneuraminic acid (Neu5Gc) and galactose-α1,3-galactose (α-gal), even though these occurring at very low levels (e.g < 2% and < 0.2% respectively) (Bosques et al., 2010; Ghaderi et al., 2010; Dietmair et al., 2012b; Ghaderi et al., 2012). The human immune system can produce antibodies against these *N*-glycans that could contribute to immunogenicity/neutralization of the corresponding biotherapeutics (Galili et al., 1984; Noguchi et al., 1995; Tangvoranuntakul et al., 2003; Chung et al., 2008; Macher and Galili 2008; Padler-Karavani et al., 2008; Ghaderi et al., 2010; Padler-Karavani and Varki, 2011). CHO cells also have limited ability to gamma-carboxylate recombinant proteins such as clotting factors (Kumar 2015), even though some improvements have been achieved through metabolic engineering work (Rehemtulla et al., 1993; Liu et al., 2014). Proteins requiring proteolytic processing for maturation may not always be fully cleaved and active when expressed in CHO. For example, co-expression of furin was shown to allow the production of fully cleaved and active von Willebrand factor in an industrial-scale CHO perfusion system (Fischer et al., 1995) and of the coagulation factor VIII B-domain (Demasi et al., 2016). Similarly, co-expression of proprotein convertases allowed for the efficient maturation of human bone morphogenetic protein-7 (Sathyamurthy et al., 2015).

2.2. Human cell lines

One way to favor human-like glycosylation would be to use human cell lines for recombinant protein production. This strategy would warrant that proteins harbor, if not the ideal glycosylation pattern, at least a non-immunogenic glycans (Swiech et al., 2012). The most commonly used human cell lines to manufacture glycoprotein therapeutics are the HEK293 cells and the HT-1080, respectively from human embryo kidney and fibrosarcoma origin (Rasheed et al., 1974; Graham et al., 1977). Drotrecogin alfa (Xigris®), the first therapeutic glycoprotein produced in human cells (HEK293) approved by FDA and EMA, was accepted by both agencies in 2001 and 2002 respectively. However, it was removed from the market in 2011, since it failed to show significant beneficial effects. Yet, only four biological glycoproteins were approved in the following decade by FDA and/or EMA. These four therapeutics, namely Agalsidase alfa, Epoetin delta (DYNEPO®), Idursulfase (ELAPRASE®) and Velaglucerase alfa (VPRIV®), are produced using a gene activation technology (proprietary of Shire) in HT-1080 cells (Moran, 2010). Epoeitin delta produced in HT-1080 was found to have better homogeneity of its tetra-antennary glycans, higher sialic acid content and no Neu5Gc, compared to CHO-produced erythropoietin (Llop et al., 2008). However, this product was voluntarily withdrawn for commercial reasons (Dumont et al., 2015). As for

Velaglucerase alfa, its glycoprofile has also been compared to similar products (other β-glucocerebrosidases) produced in CHO cells and carrot cells (Imiglucerase (CEREZYME®) and Taliglucerase alfa (ELELYSO®), see below). Even though these three products show diverse glycan profiles, they showed similar macrophage uptake, *in vitro* enzymatic activity, stability and efficacy (Ben Turkia et al., 2013; Tekoah et al., 2013). Notably, neutralizing anti-Imiglucerase antibodies were observed in 24% of patients, with an impact on the protein activity (Ben Turkia et al., 2013).

In 2014, a bulge of approvals for therapeutic proteins produced in human cell lines was observed, with four new FDA/EMA authorized glycoproteins. rFVIIIFc (ALPROLIX®) and rFIXFc (ELOCTATE®) are two of these proteins, used for prevention of bleeding episodes in people with hemophilia A and B. They consist of domains of FVIII and FIX proteins fused to the Fc portion of immunoglobulin G1 (IgG1) (Powell et al., 2012; Peters et al., 2013). rFVIIIFc has six tyrosine sulfation sites which are essential for its functionality. Besides, rFIXFc has γ-carboxylation of its first twelve glutamic acid residues, also important for its activity. Expressing these glycoproteins in HEK293 resulted in greater tyrosine sulfation and glutamic acid γ-carboxylation compared to CHO cells and excluded any α-gal and Neu5Gc from the manufactured products (Berkner, 1993; Kannicht et al., 2013; Peters et al., 2013; McCue et al., 2014, 2015). Dulaglutide (TRULICITY®), another Fc fusion protein used for treatment of type 2 diabetes mellitus, is produced in HEK293-EBNA1 cells and was approved in 2014. Lastly, the Human-cl rhFVIII (NUWIQ®), a replacement clotting factor for hemophilia A disorder is approved by EMA since 2014 and by FDA since 2015 (Dumont et al., 2015). It is produced in HEK293-F cell line and has shown similar glycosylation profile to the plasma-derived factor VIII, deprived of α-gal and Neu5Gc (Casademunt et al., 2012; Kannicht et al., 2013).

Some human cell lines are currently being used in preclinical and/or clinical development stages for recombinant glycoprotein production. This is the case for PER.C6 cells, which consist of human embryonic retinoblasts transformed with adenovirus type 5 E1A and E1B-encoding sequences (Havenga et al., 2008; Swiech et al., 2012). These cells are able to produce high titers of IgG without requiring amplification of the incorporated gene (Jones et al., 2003). MOR103 is a mAb directed against granulocyte macrophage colony-stimulating factor and is developed to treat patients with rheumatoid arthritis and multiple sclerosis (Behrens et al., 2015; Dumont et al., 2015). CL184 is a combination of two mAbs used against rabies virus (Marissen et al., 2005; Bakker et al., 2008). Both antibodies, produced in PER.C6 cells, are currently tested in clinical phase 1/2 (Dumont et al., 2015). The HKB-11 cell line is a fusion of HEK293S and human B-cell lines (Cho et al., 2003). It has recently showed high-level protein production and α2,3 and α2,6-sialic acid linkages (Picanco-Castro et al., 2013). Two other cell lines, the CAP (CEVEC's Amniocyte Production) cells of human amniocytes origin and the HuH-7 cells of human hepatocellular carcinoma origin, are presently tested for recombinant glycoprotein production in preclinical phases and both display human-like glycosylation profiles (Schiedner et al., 2008; Wolfel et al., 2011; Enjolras et al., 2012; Swiech et al., 2012; Wissing et al., 2015).

2.3. Other mammalian (non-human) cell lines

Baby Hamster Kidney (BHK) cells are mostly been used for the production of vaccines (Durocher and Butler, 2009). Only two marketed recombinant glycoproteins are currently manufactured in these cells, Factor VIIa (NovoSeven®) and Factor VIII (Kogenate® and Kovaltry®), which are other clotting factors (Durocher and Butler, 2009; Dumont et al., 2015). These large glycoproteins are abundantly glycosylated and sulfated and are thus challenging to manufacture (Soukharev et al., 2002; Ishaque et al., 2007; Nivitchanyong et al., 2007).

Murine myeloma cells (NS0 and Sp2/0), derived from tumor cells that no longer produce their original immunoglobulins, also are being used to produce some commercial monoclonal antibodies such as Cetuximab (ERBITUX®) and Palivizumab (SYNAGIS®) (Barnes et al., 2000; Dumont et al., 2015). In 2015, three new therapeutic monoclonal antibodies produced in murine cells were approved by the FDA, namely Dinutuximab (UNITUXIN®), Necitumumab (PORTRAZZA®) and Elotuzumab (EMPLICITI®), all used to treat different cancer types. Murine cells can also produce α-gal and Neu5Gc at considerably higher levels than hamster cells, increasing the risks of immunogenicity (Tangvoranuntakul et al., 2003; Chung et al., 2008; Macher and Galili, 2008; Padler-Karavani et al., 2008; Ghaderi et al., 2012).

2.4. Non-mammalian cell lines and other expression systems

While a major trend in the last decade has been to use mammalian cell lines to manufacture recombinant glycoproteins, it is not to be forgotten that there are still a large number of recombinant biotherapeutics produced in other expression systems. However, these organisms do not have the ability to adequately glycosylate recombinant proteins, due to the absence of the required enzymatic machinery. These systems are thus mainly limited to the expression of non-glycosylated proteins. Bacterial expression systems have rapid cell growth and high yields, but proteins often aggregate and have to be extracted from inclusion bodies, due to the absence of chaperone proteins, before being refolded *in vitro*. Still, some commercially available enzymes that are not glycosylated, such as asparaginase and collagenase, are produced in bacterial expression systems (Graumann and Premstaller, 2006; Ghaderi et al., 2012). Some recombinant proteins are produced in yeast, which can also divide rapidly and generate high yields. However, these cells produce glycoproteins with high-mannose glycans structures, which may be immunogenic and less potent in humans (Dean, 1999; Gemmill and Trimble, 1999; Gerngross, 2004; Dumont et al., 2015). Examples of approved therapeutics from yeast expression system are ocriplasmin (JETREA®) and catridecagog (TRETTEN®) (Dumont et al., 2015). As for plant and insect cells, both are able to produce recombinant proteins with complex glycans, but with structures quite different from the human ones. Indeed, plants produce core α1,3-fructose and β1,2-xylose, which are completely absent in human cells and could be immunogenic (Karg et al., 2009; Ghaderi et al., 2012). Insect cells produce *N*-glycan precursors that are trimmed, creating high mannose or paucimannose structures (Kost et al., 2005). Both plant and insect cells lack sialic acid residue on their glycans. Several attempts have been made to glycoengineer plant and insect cells for protein productions (Cox et al., 2006; Misaki et al., 2006; Paccalet et al., 2007; Schahs et al., 2007; Sourrouille et al., 2008; Strasser et al., 2008). In 2012, a first plant-generated therapeutic recombinant protein, taliglucerase alfa (ELELYSO®) was approved by the FDA (Walsh, 2014). Currently, the only therapeutics approved for insect cell expression systems are the human papillomavirus vaccine (CERVARIX®), the prostate cancer immunotherapy vaccine (PROVENGE®) and the flu vaccine (FLUBLOK®) (Cox, 2012; FDA, 2013; Dumont et al., 2015). Lastly, a few therapeutic proteins are produced in transgenic animals. As with other mammalian expression systems, transgenic animals often produce different glycosylation pattern compared to native human proteins (Durocher and Butler, 2009). The first therapeutic produced in transgenic animal available on the market was the human anti-thrombin alpha (ATryn®), made in transgenic goat milk (Kling, 2009). This product was followed by the C1-esterase inhibitor (Ruconest®) produced in rabbit milk, approved by the EMA in 2011 and by the FDA in 2014 (Walsh, 2014). Finally, a third product generated in transgenic chicken eggs has been approved in 2015 by both EMA and FDA, a recombinant human lysosomal acid lipase (Kanuma®).

3. Cell engineering

In the biopharmaceutical industry, production of glycoproteins is

currently achieved by either transient or stable gene expression in the cells. When the need for a quick and economical approach prevails, transient expression remains the best choice for protein production (Rosser et al., 2005; Pham et al., 2006; Geisse, 2009; Chahal et al., 2011; Baldi et al., 2012). By skipping the lengthy selection process for the cells that have integrated the plasmid within their genome, transient transfection is much faster (Rita Costa et al., 2010). However, the production rate relies on many factors including efficiency of the transfection phase, cytotoxicity of the transfection reagent and extensiveness of the feeding strategy applied to the culture post-transfection (Pham et al., 2006; Daramola et al., 2014). Although the level of protein obtained is not as high as with stable gene expression, it is still sufficient for many applications. Indeed, during transient transfection, the plasmid DNA is mostly kept extrachromosomally, the cells rapidly losing it during division, therefore limiting the amount of expressed proteins. Yet, it is ideal for high throughput screening for hits identification and very helpful for early stage product characterization (Ozturk and Hu, 2005; Rosser et al., 2005; Rita Costa et al., 2010). To date, only viral vectors used in gene therapy have been produced by transient transfections for clinical applications (Wright, 2009). When it comes to production of glycoproteins in large quantities, stable gene expression systems remain the preferred avenue. For these systems, multiple aspects have been tackled and optimized for improving productivity, process robustness and reducing cell line generation timelines (Fig. 2).

3.1. Selection systems

Many selection systems have been developed over the years to improve the production rate of the proteins and the efficiency of the selection. For stable expression, a gene marker is usually integrated in the expression plasmid along with the cDNA encoding the gene of interest, conferring a selective advantage to the cells that integrate the plasmid into their genome (Rita Costa et al., 2010). Copy number of integrated plasmid as well as the integration site(s) within the host genome are some of the key factors when it comes to stable gene expression. There are two frequently used selection markers in the biopharmaceutical industry, namely the glutamine synthetase (GS) and the dihydrofolate reductase (DHFR) genes. The first one, originally developed by Celltech (now Lonza Biologics), uses complementation of a glutamine auxotrophy by a recombinant GS gene (Fan et al., 2013). NS0 and Sp2/0 cell lines express insufficient endogenous levels of GS to support growth, therefore simply removing glutamine from the culture media is sufficient for selection. However, for CHO cells, it is necessary to add methionine sulfoximine MSX (an analogue of glutamate) in combination with the removal of glutamine in the media, to help inhibit endogenous glutamine synthetase activity and have enough selection pressure. Moreover, CHO GS-Knock out (KO) cell lines were recently developed (Eli Lilly), thus enhancing the stringency of this selection system (for further details see Section 3.2). Similarly, the DHFR selection uses the fact that CHO cell mutants have been selected to be deficient for DHFR (Urlaub and Chasin, 1980). Using a recombinant DHFR gene in the integrated plasmid, cells are put under selection pressure and gene amplification with increasing concentrations of methotrexate to inhibit the DHFR enzyme activity, combined with the absence of nucleotide precursors in the media. Other selection systems have been developed more recently, such as the OSCAR™ system from the University of Edinburgh, that uses minigene vectors encoding the hypoxanthine phosphoribosyltransferase (HPRT), essential for purine synthesis (Melton et al., 2001; Costa et al., 2012). Yet, only the DHFR and the GS systems have been used so far by the industry for high-scale commercial productions.

3.2. Gene expression

Despite the success of the selection systems described above, a main problem persists with these methods, being the fact that they are based on random plasmid integration and its expression cassette in the host genome. This random integration creates very heterogeneous cell population that have variable expression levels amongst the different clones. Transgenes are likely to be inserted in heterochromatin regions, which results in very weak gene expression levels. This implies screening a very large number of clones (generally multiple hundreds to a few thousands, depending on the cloning method used) in order to find

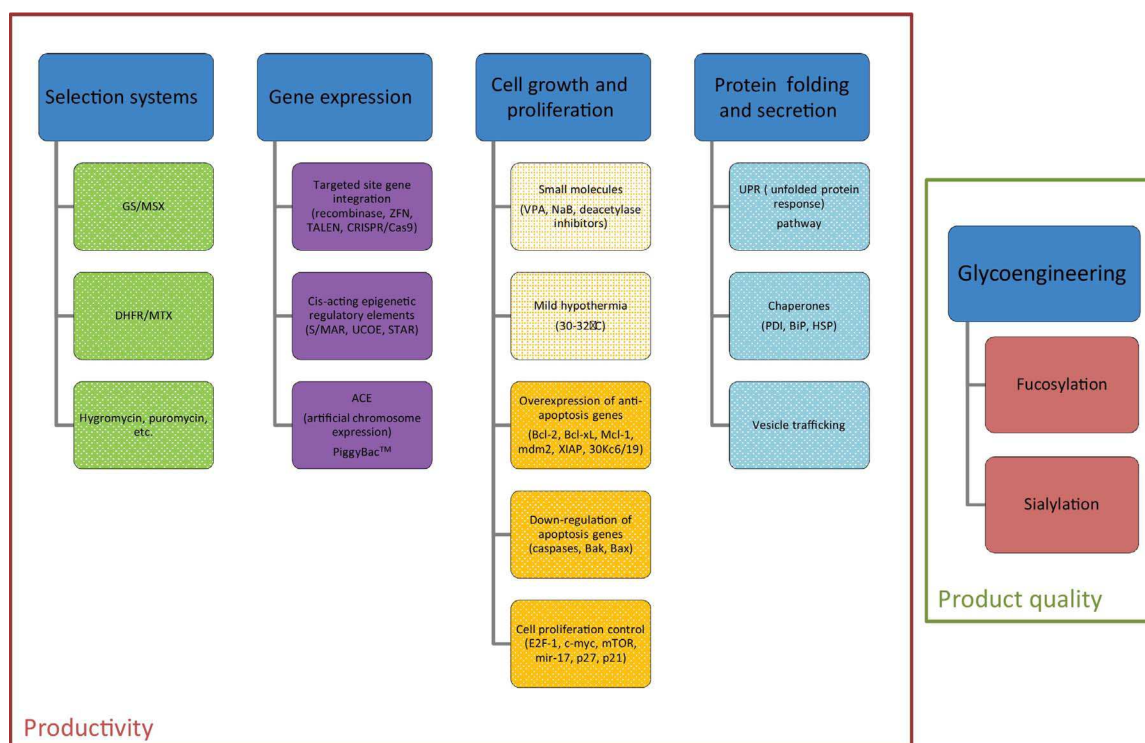

Fig. 2. Cell engineering strategies developed to improve productivity and glycosylation.

those rare ones with stably integrated plasmid in highly transcriptionally active chromatin regions (“hotspots”). Recently, several molecular and cellular biology tools have been developed for targeted site gene integration. This approach could eventually help biopharmaceutical companies minimize the randomness of gene insertion and increase the predictability for high transgene expression (Estes and Melville, 2014). Although tremendous efforts are required to isolate a highly productive and stable recombination host cell line compared to random integration strategies, it remains a very attractive avenue that should help control and predict the expression level of the daughter clones (Estes and Melville, 2014). Since the transgene and selection marker are on the same plasmid, their co-integration within the same chromatin region increases the odds that good expression of the marker will also result in good transgene expression. As first generation tools, several recombinases have been used for targeted site approaches, among which the Cre/Lox and the Flp/FRT were mostly utilized (Kito et al., 2002; Huang et al., 2007; Kim et al., 2012). Recombination mediated cassette exchange (RMCE) technology is attracting interest by the industry for targeted gene insertion. It uses a site specific recombinase to exchange a cassette flanked with heterospecific sequences in a plasmid with a cassette flanked by identical sequences within the host genome. This technology had good success in increasing the success rate and reducing timelines for the generation of stable industrial-grade CHO cell lines stably expressing monoclonal antibodies (Kito et al., 2002; Huang et al., 2007; Zhang et al., 2015).

As second generation tools, different endonucleases have been employed. This category includes zinc finger nucleases (ZFN), meganucleases, transcription activators like effectors nucleases (TALEN) and CRISPR/Cas9 (Durai et al., 2005; Porteus and Carroll 2005; Cabaniols et al., 2010; Silva et al., 2011; Kim et al., 2012; Sanjana et al., 2012; Cho et al., 2013; Sun and Zhao, 2013). These nucleases induce DNA double-strand breaks at precise locations into the host genome, facilitating the integration of the cassette by non-homologous end-joining (NHEJ) or homology-directed repair (HDR) (Gaj et al., 2013). Both ZFN and TALEN technologies rely on the ability to customize a DNA-binding domain for a specific sequence (the targeted sequence for cleavage) combined to a nuclease effector domain. These nucleases are thus dependant on the ability of developing a good DNA recognition motif, with high specificity and affinity. Because the genome contains numerous sequence repetitions or has highly homologous DNA sequences, efforts have been made in the last years to improve the off-target effects and the specificity of these enzymes (Miller et al., 2007; Szczepek et al., 2007; Guo et al., 2010; Doyon et al., 2011). As an efficient alternative to these protein-based genome editing nucleases, the CRISPR/Cas9 technology has just emerged. It relies on a RNA-guided cleavage with a CRISPR RNA that will bind to a specific DNA sequence (seed) combined with the Cas9 endonuclease (Wiedenheft et al., 2012; Cho et al., 2013; Gaj et al., 2013). This technique has already been validated in CHO cells and reduces the production variability between clones (Lee et al., 2015b). ZFN, TALEN and CRISPR/Cas9 technologies are however mostly used for gene-specific knockout. The ZFN knockout approach was particularly successful for specific deletion of the GS and the DHFR genes in CHO cells, thus improving the selection stringency of the generated cell lines (Liu et al., 2010). Further applications related to gene knockout for glycoprotein production will be discussed in the glycoengineering section. Even if these tools could prove very useful for site specific integration, one of the key challenges is still to identify good hotspots in the host genome that will allow good expression levels and stability (Zhang et al., 2015). One should also keep in mind that such specific integration may not be a one-size-fits-all approach as some therapeutic proteins may require a particular level of expression to fold properly, or to acquire adequate quality attributes (e.g. glycosylation, proteolytic processing, etc.)

Another, but less commonly used tool, is the mammalian artificial chromosome expression (ACE) technology. This minigenome serves as an autonomous genetic element that replicates with the cells. Its DNA sequence is customizable with various regulating elements that could possibly help for its expression (Lai et al., 2013; Estes and Melville 2014). It was shown to be effective for establishing CHO cell lines producing respectable titers of monoclonal antibody (Lindenbaum et al., 2004; Kennard et al., 2009; Kennard 2011). Fed-batch performance and stability of ACE vs random integration systems have been compared for IgG1 expression in CHO cells and have shown similar performance (Combs et al., 2011). Also, the PiggyBac™ (System Bioscience inc.) transposon system uses an efficient transposase purified from the cabbage looper (*Trichoplusia ni*) to easily integrate the gene of interest into the host genome (Ding et al., 2005). This approach has recently shown improved yields for stable production of antibodies in CHO cell lines (Rajendra et al., 2016).

Some cis-acting epigenetic regulatory elements have also been developed to improve the production level and stability of the producing cell lines. These elements help remodelling the chromatin environment to maintain an active transcriptional state around the transgene. One of the most frequently used cis-acting elements is the scaffold/matrix attachment region (S/MAR). Many reports have shown that including these MAR elements in expression vectors for recombinant proteins could significantly improve the expression levels (for an extensive review see (Harraghy et al., 2015)). However, conflicting results in the literature have led to the conclusion that MAR activity could be sequence-specific and is influenced by specific vector configuration (Harraghy et al., 2015). In recent years, development of bioinformatic tools capable to predict the outcome of MAR sequences helped to improve MAR elements (Girod et al., 2007; Harraghy et al., 2012, 2015). Another class of epigenetic regulatory elements is the ubiquitously acting chromatin opening elements (UCOEs). These sequences contain CpG rich islands found within the promoter regions of housekeeping genes which confer an open chromatin state for transgene expression. UCOEs were also found to be helpful for increasing the productivity of recombinant protein cell lines (Benton et al., 2002; She et al., 2009). The third class of epigenetic elements is the stabilizing and anti-repressor (STAR) element. They were discovered by a genomic screen of elements that would increase transgene expression. (Kwaks et al., 2003; Sautter and Enenkel, 2005; Hoeksema et al., 2011; Van Blokland et al., 2011; Harraghy et al., 2015). However, the use of STAR elements has not been reported for industrial production.

Inclusion of these various epigenetic elements not only can help to increase the expression level of biotherapeutics, but can also increase the number of clones that have integrated the transgene with a more defined copy number of transgene per cell, thus accelerating the selection process (Grandjean et al., 2011; Harraghy et al., 2015). It was also demonstrated that MAR and UCOE helped decrease the variability of expression between the different clones and contributed in maintaining an active transcriptional state for better transgene expression (Williams et al., 2005; Galbete et al., 2009; Harraghy et al., 2011). It has been shown that an UCOE can also prevent DNA methylation of the CMV promoter region, thus preventing gene expression silencing (Lindahl Allen and Antoniou, 2007; Zhang et al., 2010a). Since most of these elements are composed of nucleosome depleted regions, this could also contribute to the inhibition of the propagation of an inactive chromatin state surrounding the transgene, serving as genetic boundaries (Arope et al., 2013; Harraghy et al., 2015).

3.3. Cell growth, proliferation and survival

Producing more proteins with fewer resources and in less time remains a significant challenge when working with mammalian cell expression systems. In this perspective, various tools and methods focusing on cell growth, proliferation and survival have been tackled by the biopharma industry and academia. Key aspects for the optimization of cell growth and productivity certainly are the process parameters (pH, temperature, mixing, etc.), as well as the media and the feeds

composition. These media and feeds should maintain availability of key nutrients and avoid accumulation of metabolites having negative impact on the culture performance (see (Andersen et al., 2009) for further details). However, the composition of most of the commercial media and feeds are industrial trade secrets, rendering their optimization resource-intensive. Beside media and feeds, numerous small molecules have been identified from a chemical library that could enhance protein production (Allen et al., 2008). Sodium butyrate (NaB) and valproic acid (VPA) are often used in cell culture media. These histone deacetylase inhibitors, by preserving histones acetylation, reduce their interaction with DNA and helps maintain chromatin in an open configuration, leading to enhanced or prolonged transgene transcription. Addition of these chemicals has resulted in increased productivity in numerous studies, especially in CHO cells (Durocher and Butler, 2009; Yang et al., 2014). However, the use of these inhibitors may also have undesired secondary effects, such as blocking cell cycle or inducing apoptosis (Sung et al., 2004; Jiang and Sharfstein, 2008; Sunley and Butler, 2010). To attenuate these effects, optimization of the addition timing and concentration has shown to be important (Rodriguez et al., 2005). Moreover, combining these inhibitors with mild hypothermia (30–32 °C) during production phase or with anti-apoptosis cell engineering could also increase productivity (Kim and Lee, 2000; Kim and Lee, 2002; Sung et al., 2007; Kim et al., 2009a; Chen et al., 2011).

These hypothermia and anti-apoptosis strategies have been used to extend cell survival of CHO cells and thus maximize productivity. Indeed, simply using reduced temperature during protein production phase in CHO cells significantly improved the yield of numerous model proteins such as alkaline phosphatase, erythropoietin, interferon-gamma and beta-interferon by slowing down cell cycle progression and allowing cells to shift from a proliferative to a productive mode (Kaufmann et al., 1998; Yoon et al., 2003; Fox et al., 2004; Ahn et al., 2008; Sunley et al., 2008). This shift toward protein production favors sustained nutrient availability and reduced secondary metabolite accumulation. As for anti-apoptosis engineering, genes of the Bcl-2 family (Bcl-2, Bcl-xL and Mcl-1) were overexpressed in a variety of CHO cell lines (Kim and Lee, 2000; Tey et al., 2000; Meents et al., 2002; Chiang and Sisk, 2005; Kim et al., 2009b; Majors et al., 2009). Oppositely, down-regulation of pro-apoptotic genes like Bax, Bak, microRNA mir-mmu-miR-466h-5p and caspase-3,-7,-8 and −9 were also tested (Kim and Lee, 2002; Sung et al., 2007; Yun et al., 2007; Cost et al., 2010; Druz et al., 2013). All strategies resulted in increased protein production. Successful apoptosis inhibition was also achieved by overexpression of other related anti-apoptotic proteins (Durocher and Butler, 2009; Bandaranayake and Almo, 2014). Although the effects on cell longevity are clear in these studies, many conflicting results regarding enhanced productivity with anti-apoptosis engineering have been reported (Kim et al., 2012).

Some engineering strategies rather rely on cell proliferation control. For example, the E2F-1 cell cycle transcription factor overexpression contributes to increase viable cell density in CHO batch cultures (Majors et al., 2008). The CDKL3 gene insertion also accelerates cell proliferation of 293 and CHO cells (Jaluria et al., 2007). Cell cycle progression was also faster when using c-myc oncogene in CHO cells (Kuystermans and Al-Rubeai, 2009). The mTOR pathway, involved in cell proliferation, survival and translation has also been exploited. The introduction of the mTOR gene in CHO cells resulted in increased cell proliferation and productivity of therapeutic IgG, alkaline phosphatase and α-amylase (Dreesen and Fussenegger, 2011). Moreover, chemical manipulation of this pathway can also enhance glycoprotein productions in these cells (Dadehbeigi and Dickson, 2015). Furthermore, overexpression of the mir-17 microRNA in CHO was shown to augment cell proliferation speed, leading to higher Epo-Fc yield (Jadhav et al., 2012).

3.4. Protein folding and secretion

Another limitation of glycoprotein production is the proper folding of the desired biologics. As these proteins are being translated, they travel through the endoplasmic reticulum (ER) and the Golgi for proper glycosylation and folding before being secreted. It has been suggested that translational and/or post-translational processes might also be rate-limiting for protein production, because of the lack of correlation between gene copy number and the amount of secreted proteins (Wilson et al., 1990; Mohan et al., 2008). However, playing with the level of ER proteins involved in the secretory pathway has shown mixed results, depending of the expression system and/or the overexpressed protein. Some reports have revealed positive effects in overexpressing XBP-1 (X-box binding protein 1), a transcription factor regulating the unfolded protein response (UPR) during ER stress (Iwakoshi et al., 2003; Tigges and Fussenegger, 2006; Becker et al., 2008; Ku et al., 2008). However there were no obvious benefit observed in stable CHO-K1 cells expressing IFNy, EPO or a human mAb (Ku et al., 2008). Also, in response to ER stress, cells normally attenuate their translational machinery. ATF4, another critical transcription factor in the UPR system, has been overexpressed to restore translation and enhance levels of anti-thrombin III produced in CHO cells (Ohya et al., 2008). This activation is made through the GADD34 protein, which recruits the PP1 phosphatase responsible for eIF-2A translation initiation factor dephosphorylation and activation. Similarly, GADD34 can also be overexpressed in order to increase productivity of CHO cells (Omasa et al., 2008). Additionally, secretory bottlenecks of CHO cells may be relieved by overexpressing soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs). For example, by controlling vesicle docking and fusion, expression of SNAP-23 and VAMP8 has been shown to improve production of SEAP and monoclonal antibodies in CHO-K1 cells (Peng et al., 2011). Similarly, overexpression of ceramide-transfer protein (CERT) S132A mutant, which is responsible for the transfer of ceramide from the ER to the Golgi and for sphingomyelin synthesis, also improved production of t-PA, human serum albumin and monoclonal antibodies in industrial cell lines (Florin et al., 2009; Rahimpour et al., 2013). Likewise, signal recognition particle 14 (SRP14), which targets secreted proteins to the endoplasmic reticulum membrane, enhanced productivity of recombinant IgG in CHO cells when overexpressed (Lakkaraju et al., 2008; Le Fourn et al., 2014).

Chaperones play a key role in the folding process of nascent proteins. They have been employed by many groups as cell engineering tools but have given variable results (Mohan et al., 2008). The most popular chaperone for CHO cell engineering is the protein disulfide isomerase (PDI), which catalyzes the formation of disulfide bonds in proteins. Its overexpression has shown positive results on productivity in the case of monoclonal antibodies in both CHO cells and NS0 cells (Smales et al., 2004; Mohan et al., 2007). However, other studies observed either a negative or no effect on productivity(Mohan et al., 2008). Similarly, the heavy chain-binding protein (BiP) chaperone gave mixed results, with enhanced or decreased productivity (Mohan et al., 2008). Moreover, overexpression of the endoplasmic-reticulum ERp57 protein, an isoform of PDI, together with calnexin/calreticulin was able to increase thrombopoietin production in CHO cells (Mohan et al., 2008). Overexpression of the Hsp27 and Hsp70 chaperones in CHO cells also increased recombinant IFNγ production (Lee et al., 2009). To explain such discrepancies between studies, it was recently determined that CHOK1 hosts have larger endoplasmic reticulum and higher mitochondrial mass compared to DUXB11-derived CHO cells (Hu et al., 2013). Thus, these various and sometimes contradictory results could be partly explained by the use of different CHO host strains.

Other cellular pathways have been engineered to sustain cell productivity. Among these approaches, anti-autophagy engineering and metabolic engineering have been used in order to optimize recombinant protein production in mammalian cells (for a detailed review on the topic see (Kim et al., 2012; Kim et al., 2013)). Yet, most of

these strategies still need to prove their effectiveness and robustness for increasing recombinant protein production in mammalian cells, especially at an industrial level.

4. Glycoengineering

As mentioned previously, the expression systems used for glycoprotein productions have significantly different glycosylation machineries. Plants, yeasts and non-human cell lines can generate glycans that are often absent from endogenous human proteins (see section 2). For example, yeasts and insect cells can produce high-mannose or paucimannose oligosaccharides, while plant cells introduce undesirable α1,3-fucose and β1,2-xylose. Moreover, as opposed to human cells, CHO and BHK cells do not express any α2,6-sialyltransferase activity and can generate glycans with terminal Neu5Gc and α-gal. However, both Neu5Gc and α-gal levels are significantly higher in murine cells such as NS0 and Sp2/0, compared to hamster cells. To avoid any potential immunogenic response, glycans present on biotherapeutic proteins should be compatible for human hosts. Over the years, many studies have helped elucidate the biological functions of protein glycosylation. From these studies, specific glycosylation profiles have been identified, which could increase stability and efficacy of therapeutics. Consequently, several glycoengineering strategies have emerged to recreate these beneficial profiles on recombinant proteins. In particular, afucosylation and sialylation strategies are now popular glycoengineering approaches for biologics.

4.1. Reducing fucosylation

It was reported that afucosylated Fc domain glycans of human IgG1 could enhance the binding to FcγRIIIa subtype receptors of natural killer cells (Shields et al., 2002; Shinkawa et al., 2003; Niwa et al., 2005; Iida et al., 2006; Subedi and Barb, 2016). Recently, the same effect was observed for human IgG4 (Gong et al., 2016). This increased affinity allows better therapeutic activity of Mabs used in cancer therapy, by triggering the antibody-dependent-cell-mediated cytotoxicity (ADCC) pathway (Niwa et al., 2005; Iida et al., 2006; Chung et al., 2012). Since mAbs represent by far the most important class of therapeutic glycoproteins being manufactured, research towards development of strategies to produce low or non-fucosylated Mab glycans has intensified. As early strategies, CHO Lec13 cells and rat hybridoma YB2/0 cells were employed, the former for its impaired synthesis of GDP-fucose and the latter for its reduced expression level of the fucosyltransferase FUT8 (Durocher and Butler, 2009). Since then, some FUT8 knock-out CHO cell lines have been established and one is currently available on the market for glycoprotein productions. The first FUT8$^{-/-}$ cell line was created by sequential homologous recombination (Yamane-Ohnuki et al., 2004). Recently, both ZFN and a CRISPR/Cas9 approaches were also used for FUT8 gene-specific knockout (Malphettes et al., 2010; Ronda et al., 2014). Disruption of the fucosyltransferase in these cell lines completely abolished the fucosylation on the Fc domain of IgG. Other methods to reduce the fucosylation level of antibodies glycan were also examined. Since expression of the β1,4-mannosyl-glycoprotein 4-β-*N*-acetylglucosaminyltransferase III (GnTIII) blocks fucosylation, CHO cells were engineered with both GnTIII and Golgi mannosidase II (ManII) for IgG production (Davies et al., 2001; Ferrara et al., 2006). Others have tried to reduce the fucosyltransferase level using siRNA (Imai-Nishiya et al., 2007). Heterologous expression of the bacterial enzyme GDP-4-dehydro-6-deoxy-D-mannose reductase (RMD) in CHO cells strongly reduced their fucosylation capacity (von Horsten et al., 2010). Lastly, other expression systems have been used to produce non-fucosylated antibodies. For example, a Pichia pastoris yeast strain was engineered to generate human-like glycans but devoid of core-fucose (Choi et al., 2003; Hamilton et al., 2006; Hamilton and Gerngross 2007). Consequently, the anti-CS1 antibody produced with these cells is afucosylated and has enhanced *in vitro* ADCC activity and *in vivo* anti-tumor activity compared to the same antibody produced in HEK293 cells (Gomathinayagam et al., 2015).

4.2. Increasing sialylation

Addition of terminal sialic acids on the glycans of protein therapeutics helps to maintain them into the blood circulation by preventing their recognition by the asialoglycoprotein receptors highly expressed in liver hepatocytes. Thus, increasing the sialylation of proteins may decrease the frequency of injection or the amount of therapeutic protein used in a single dose. Therefore, it has become attractive for the industry to produce proteins with optimal sialylation as it could generate substantial savings and confer a therapeutical advantage for the patients. Since the sialic acid pathway includes many steps, various glycoengineering strategies have been elaborated in order to improve sialylation of biologics. Some of these approaches are based on the supplementation of cell culture media with different precursors of the sialic acid pathway, such as CMP-sialic acid, ManNAc and NeuNAc, but their effects are limited and still debated (Gu and Wang, 1998; Baker et al., 2001; Hills et al., 2001; Bork et al., 2009; Hossler et al., 2009; Wong et al., 2010; Kildegaard et al., 2016). Transient expression of different sialylation machinery enzymes was also tested. A functional analysis of 31 glycosyltransferases was even conduced for human EPO glycosylation optimization in six different mammalian cell lines, where enhanced expression of ST3GalII, ST3GalIV and ST6Gal1 could enhance sialylation in HEK293, Cos-7, 3T3 and NSO cells (Zhang et al., 2010c). Transient expression of ST6Gal1 in CHO and HEK293 cells also increased the α2,6 sialylation of a trastuzumab F243A mutant antibody (Raymond et al., 2012). Co-expression of ST6Gal1 and β4GalT1 in CHO cells also significantly enhanced the sialylation level of the mutated version of trastuzumab (Raymond et al., 2015). Co-expression of the same enzymes in HEK293 also helped optimizing sialylation of human Fc-IVIg (intravenous immunoglobulin), therefore enhancing its therapeutic activity (Washburn et al., 2015). Finally, transient transfection of the Chinese hamster ST6Gal1 gene in CHO cells expressing a bispecific antibody, also considerably increased the level of α2,6 sialylation (Onitsuka et al., 2012).

As genome-editing strategies, the ST6Gal1 gene of the Chinese hamster was also recently used in stable expression of different CHO cell lines producing recombinant IgGs (Lin et al., 2015). Pools were enriched based on FITC-labelled *Sambucus Nigra* (SNA) lectin staining, a lectin which mainly recognizes terminal α2,6 sialic acid, and best clones were selected based on their sialylation content. An engineered CHO cell line stably expressing the human α2,3-sialyltransferase ST3Gal3, the rat mutated GNE/MNK-R263L-R266Q glycosylation enzymes and the Chinese hamster CMP-sialic acid transporter was successful in increasing tetra-sialylation of recombinant human EPO (Son et al., 2011). As mentioned earlier, the silkworm hemolymph anti-apoptotic protein 30Kc19 was shown to increase protein production (Wang et al., 2011). A CHO cell line stably expressing both 30Kc19 and human EPO showed that it could also significantly increase the EPO sialylation (Wang et al., 2011). In an effort to characterize the contributions of the various glycosylation enzymes in CHO-K1 cells, knockouts of 19 glycosyltransferase genes were recently achieved and EPO glycosylation was characterized to measure their impact (Yang et al., 2015). This study revealed predominant roles for some enzymes into CHO cells glycosylation pathway. Noteworthy, knockout of both ST3gal4 and ST3gal6 genes affected sialylation. Additionally, the same study presented a human ST6Gal1 knock-in experiment in ST3Gal4/6 knockout CHO cells, which resulted in considerable increase of α2,6 sialylation. Furthermore, to improve *N*-glycan homogeneity on therapeutic proteins, a technique called "GlycoDelete" engineering was also recently developed in HEK293S cells (Meuris et al., 2014). This system consists of engineered 293SGnTI(−) cells stably expressing the catalytic domain of endo-β-*N*-acetylglucosaminidase from the fungus *Hypo-*

crea jecorina fused to the Golgi targeting domain of human ST6Gal1. Using this expression system for transient anti-CD-20 production, recombinant proteins had very short sialylated *N*-glycans with high homogeneity on the Fc domain and reduced affinity to FcγRs compared to proteins expressed in wild-type 293S. This reduced affinity might be required to increase safety, for example in the context of neutralizing antibodies used for inflammatory cytokine targeting (Lux et al., 2013). Finally, there are also transgenic strategies to improve sialylation for other expression systems. The tobacco plant *Nicotiana benthamiana* expression system was genetically modified to carry the human-type sialylation pathway and to generate mono- and disialylated structures with α2,3 and α2,6 linkage (Kallolimath et al., 2016).

In order to increase glycoprotein sialylation levels, strategies to inhibit sialidases have also been employed. The mammalian genomes possess 4 sialidase genes or pseudogenes (depending on the specie) which are expressed in various cellular compartments (Warner et al., 1993; Ferrari et al., 1994; Zhang et al., 2010b). Many chemical inhibitors have been synthesized to target these enzymes but their use in large-scale bioprocess productions is still very limited due to their elevated fabrication cost (Ferrari et al., 1998). Some attempts of knocking down the different CHO sialidases by shRNA or siRNA also improved the sialylation level of recombinant hIFNγ, particularly for Neu2 and Neu3 sialidases (Ngantung et al., 2006; Zhang et al., 2010b).

Lastly, there are also *in vitro* glycoengineering platforms which aim at optimizing the sialylation of therapeutics. Cst-II, a bacterial sialyltransferase that can catalyze α2,8 sialylation, allows efficient polysialylation by the bacterial polysialyltransferase on recombinant alpha-1-antitrypsin *in vitro* and this results in a significantly longer half-life in mice, without affecting its specific activity (Lindhout et al., 2011). Besides, *in vitro* galactosylation and sialylation of IgG1 Fc glycans did not show any impact on ADCC activity but showed a slight improvement of binding to FcγRIIa (Thomann et al., 2015).

5. The « Omics » perspective

The publication of the CHO-K1 genome sequence in 2011, followed by the publication of two Chinese hamster and six CHO cell line genomes in 2013, bring new opportunities in developing and engineering CHO cells for improved glycoprotein production (Xu et al., 2011; Brinkrolf et al., 2013; Lewis et al., 2013). The biopharmaceutical industry has entered the "omics" era, where systems biology is a central concept (Kildegaard et al., 2013). Genomics, transcriptomics, proteomics and metabolomics are now some examples of the available tools to enhance protein production in CHO cell factories. Sequencing of the CHO-K1 genome allowed the identification and positioning of their genes and of most of human glycosylation-associated transcripts (Xu et al., 2011). This information, combined with advances of in deep-sequencing and genome-editing technologies such as ZFN or CRISP/Cas9, now facilitate genome manipulations for cell engineering strategies. As an example, CHO cells, where simultaneous disruption of FUT8, BAX and BAK gene by CRISP/Cas9 was achieved and confirmed by deep sequencing, showed increased resistance to apoptosis (Grav et al., 2015). (For a detailed review of engineering strategies using the CRISP/Cas9 technology for genome edition in CHO or human cell lines see (Lee et al., 2015a). Moreover, these omics technologies will certainly help for cGMP compliant processes and biomanufacturing, as they will facilitate extensive characterization of engineered cell lines. For example, deep-sequencing of knock out cell lines can now be achieved to determine their exact sequence and to ensure their stability in terms of genomic rearrangements (Kremkow and Lee, 2013).

In a next generation sequencing (NGS) study regrouping diverse cDNA libraries from CHO cell lines, 29 000 transcripts were assembled, which identified 13 187 mRNA transcripts (Becker et al., 2011). Combined with previous NGS studies in various CHO cell lines, this study was determinant in establishing the transcriptome profile of these cells (Birzele et al., 2010; Jacob et al., 2010). So far, the analysis of transcriptomic data has already helped finding chromosomal regions or genes influencing cell productivity. Comparison of gene expression profiles between high and low producing CHO clones allowed the identification of a deletion in chromosome 8 telomeric region of the cells correlating with higher productivity (Ritter et al., 2016b). The C12orf35 gene comprised within this region seems to be responsible for the phenotype (Ritter et al., 2016a). Moreover, with the help of transcriptomic tools such as RNA-seq, microRNA expression profiles of CHO cells are now available (Hackl et al., 2011; Johnson et al., 2011; Hammond et al., 2012; Kozomara and Griffiths-Jones, 2014). With growing evidence for their role in diverse biological processes, some microRNA genes serve as novel targets for cell engineering strategies (Muller et al., 2008; Jadhav et al., 2013). Higher recombinant protein production was obtained when overexpressing the miR-7 gene in CHO cells (Barron et al., 2011). CHO cell lines producing recombinant IgG were also found to have decreased expression of miR-221 and miR-222 compared to their parental cell line DG44 (Lin et al., 2011). Also, as discussed earlier, inhibition of the mmu-miR–466 h microRNA was shown to increase apoptosis resistance of these cells (Druz et al., 2011; Druz et al., 2013).

The CHO genomic sequence also contributed to a surge in proteomic studies. A first large-scale proteomic analysis identified 6164 proteins from both the proteome and glycoproteome of CHO-K1 cells (Baycin-Hizal et al., 2012). This multidimensional study also resolved the codon frequency in these cells, which largely contributed to sequence optimization of genes encoding recombinant proteins for this expression system. Moreover, combination of these proteomic data with available transcriptomic data could elucidate the relative levels of the different biological pathways found within CHO-K1 cells (Kildegaard et al., 2013). Proteome analysis using SILAC (stable isotope labeling) and iTRAQ (isobaric tags for relative and absolute quantification) techniques are now widely used for biomarker identification in antibody-producing cells (Kildegaard et al., 2013). Such biomarkers contribute in targeting specific proteins for cell line engineering. Using iTRAQ, MCM2 and MCM5 helicases were identified as cell growth markers in CHO cells (Carlage et al., 2012). Besides, chaperones BiP and PDI were differentially expressed in stationary compared to exponential phase in CHO cells overexpressing Bcl-xl (Carlage et al., 2012). Furthermore, two-dimensional gel electrophoresis and mass spectrometry techniques are both proteomic tools widely used to identify changes in protein levels under precise experimental conditions (see (Kim et al., 2012) for a detailed review). Together, these two techniques will definitely contribute to the elaboration of cell engineering strategies. Indeed, comparison of proteomic profiles of fast vs slow growing CHO clones has already identified the valosin-containing protein (VCP) as a potential candidate for enhancement of cell growth by cell engineering (Doolan et al., 2010). Other candidates will likely come out of these analyses in the near future.

In addition to genomics, transcriptomics and proteomics tools, metabolomics approaches are now used in order to optimize glycoprotein production in CHO cell lines. Recent advances in metabolite quantification have allowed identification of cellular phenotypes under specific experimental conditions (Sellick et al., 2011b). Nutrient utilization and metabolic by-products accumulation are now easily quantifiable and serve as read-outs to improve cell culture conditions. Such tools have particularly contributed to the optimization of feeding cocktails and culture media to increase recombinant glycoprotein production and extend cellular growth (Sellick et al., 2011a; Zang et al., 2011; Chong et al., 2012; Dietmair et al., 2012a; Mohmad-Saberi et al., 2013). Moreover, new targets for cell engineering approaches can be identified, based on metabolomics profiling. A bottleneck at the malate dehydrogenase II (MDHII) level was characterized for the tricarboxylic acid (TCA) cycle in CHO cells and pyruvate metabolism was shown to vary between high producing and low producing anti-CD20 CHO clones (Chong et al., 2010; Ghorbaniaghdam et al., 2014). Finally, a multi-omics study combining transcriptomics and metabolo-

mics data, identified variations in gene expression and in enzymatic reactions during the transition from a parental HEK293 cell line to a producer cell line (Dietmair et al., 2012b).

6. Conclusions and perspectives

Mammalian cell lines, in particular CHO cells, are now extensively used for production of therapeutic glycoproteins by the biopharmaceutical industry. These cells possess many advantages in terms of cell culture and have the capacity to generate high titers. Over the years, many cell engineering strategies were attempted in order to increase such titers by optimizing selection markers, gene expression, cell growth and proliferation or protein folding and secretion. Among those engineered tools, CRISPR/Cas9 and RMCE technologies will largely contribute to the advance of glycoprotein production in a near future. Besides, glycoengineering strategies have been developed to reduce fucosylation or increase sialylation of biologics. Improving the glycosylation profile of biologics will definitively continue being a priority for the industry in order to enhance their quality and bioactivity.

Furthermore, the "omics" revolution brings up new challenges, as for scientists to integrate and analyze a tremendous amount of data. Considerable efforts in data mining and in the development of modeling tools will certainly be required. Yet, combining all "omics" data using computational models will help broaden our understanding and improve the various expression systems used by the biopharmaceutical industry for glycoprotein production. These powerful tools will bring valuable contribution to the advance of research and development in biotechnology and lead to the next generation of cell factories.

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